Roles for leptin receptor/STAT3-dependent and -independent signals in the regulation of glucose homeostasis

Sarah H. Bates,1 Rohit N. Kulkarni,3 Matthew Seifert,3 and Martin G. Myers, Jr.1,2,*

1Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109
2Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan 48109
3Research Division, Joslin Diabetes Center, Harvard Medical School, 1 Joslin Place, Boston, Massachusetts 02215
*Correspondence: mgmyers@umich.edu

Summary

Leptin activates the long form of the leptin receptor (LRb) to control feeding and neuroendocrine function and thus regulate adiposity. While adiposity influences insulin sensitivity, leptin also regulates glucose homeostasis independently of energy balance. Disruption of the LRb/STAT3 signal in s/s mice results in hyperphagia, neuroendocrine dysfunction, and obesity similar to LRb null db/db mice. Insulin resistance and glucose intolerance are improved in s/s compared to db/db animals, however, suggesting that LRb/STAT3-independent signals may contribute to the regulation of glucose homeostasis by leptin. Indeed, caloric restriction normalized glycemic control in s/s animals, but db/db mice of similar weight and adiposity remained hyperglycemic. These differences in glucose homeostasis were not attributable to differences in insulin production between s/s and db/db animals but rather to decreased insulin resistance in s/s mice. Thus, in addition to LRb/STAT3-mediated adiposity signals, non-LRb/STAT3 leptin signals mediate an important adiposity-independent role in promoting glycemic control.

Introduction

The incidence of type 2 diabetes in industrialized nations has increased dramatically over the past several decades and continues to increase; much of this increase is attributable to the burgeoning incidence of obesity in these populations (Green, 1997; Hubert et al., 1983). Leptin, the product of the obese (ob) gene (Friedman and Halaas, 1998; Zhang et al., 1994), is a hormone that is secreted by adipose tissue to signal the status of body energy stores to the central nervous system (CNS; Halaas et al., 1995). As a signal of energy sufficiency, adequate leptin levels suppress feeding and regulate neuroendocrine function (Friedman and Halaas, 1998; Ahima et al., 1996, 1997; Heiman et al., 1997; Yu et al., 1997). Disrupted leptin action in ob/ob (devoid of leptin) and db/db mice (devoid of the signaling, or LRb, leptin receptor) results in hyperphagia and obesity, endocrine dysfunction, and predisposition to diabetes.

In addition to its role in regulating glucose homeostasis by controlling energy balance and thus adiposity, numerous lines of evidence suggest a direct role for CNS leptin action in regulating glucose homeostasis. Not only is the impaired glycemic control in animals with disruption in leptin or LRb action not reversed by pair feeding or fasting, but glycemic control is reasonably normal in other similarly obese rodents, such as the AV mouse (Kahn and Rossetti, 1998). Furthermore, exogenously administered leptin acutely enhances glycemic control in ob/ob mice prior to noticeable effects upon feeding or adiposity, and leptin administration improves hepatic glucose flux in wild-type as well as ob/ob mice (Liu et al., 1998; Burcelin et al., 1999; Kamohara et al., 1997; Barzilai et al., 1999). Furthermore, both in human patients and in animal models of lipodystrophic diabetes, leptin administration improves insulin sensitivity and diabetes, while food restriction does not (Petersen et al., 2002; Oral et al., 2002; Shimomura et al., 1999; Colombo et al., 2002; Ebihara et al., 2001). These leptin actions on glucose homeostasis appear to be mediated via the CNS, as similar effects are observed upon intracerebroventricular (ICV) as well as peripheral administration of leptin in rodents (Liu et al., 1998), and brain-restricted transgenic expression of LRb predominantly rescues the obesity/diabetes phenotype of db/db mice (Kowalski et al., 2001; Chua et al., 2004; Cohen et al., 2001). It has also been suggested that leptin may directly regulate insulin production by the pancreatic β cell, as incubation of primary islet cultures with leptin results in a suppression of insulin secretion (Kulkarni et al., 1997).

Leptin binding to LRb activates the associated Jak2 tyrosine kinase to initiate downstream signaling (Ihle and Kerr, 1995; Taniguchi, 1995; Kloek et al., 2002). LRb signaling can be conceived of as originating from three major sites within the activated receptor complex: from each of two tyrosine phosphorylation sites on LRb itself (Tyr1138 and Tyr1139) and from signaling motifs on the LRb-associated Jak2 molecule (Kloek et al., 2002; Bjorbaek et al., 2001; Banks et al., 2000). While signals mediated directly by motifs on the LRb-associated Jak2 are poorly characterized, it is well known that Tyr1138 recruits the binding of the protein tyrosine phosphatase, SHP-2 and the inhibitory suppressor of cytokine signaling-3 (SOCS3), while Tyr1139 specifically binds and activates the latent transcription factor, signal transducer and activator of transcription-3 (STAT3; Banks et al., 2000; Bjorbaek et al., 2000; Li and Friedman, 1999; Symes et al., 1997; White et al., 1997; Vaisse et al., 1996; Bates et al., 2003). By studying mice in which the gene for the leptin receptor (lept) was replaced with a substitution mutant at Tyr1138 (lepr1138) by homologous targeting, we have previously shown...
that disruption of the LRβ/STAT3 signal in homozygous (s/s) mice, results in hyperphagia, reduced energy expenditure, and obesity with preserved reproductive and growth function (Bates et al., 2003; Bates et al., 2004). Here we utilize this model of single pathway leptin resistance to investigate the role of the LRβ/STAT3 pathway in leptin control of glucose homeostasis. We demonstrate that while LRβ/STAT3 signaling is important for the regulation of glucose homeostasis via feeding and adiposity, LRβ/STAT3-independent signals regulate insulin sensitivity independently of effects on energy balance.

Results and discussion

Improved glucose homeostasis in s/s compared to db/db mice

We have previously shown that disruption of the LRβ/STAT3 signal in s/s mice results in hyperphagia, decreased metabolic rate/energy expenditure, and increased adiposity similar to that observed in db/db mice (Bates et al., 2003, 2004). Compared to db/db mice that become floridly diabetic, however, the alterations in glucose homeostasis in obese s/s animals are relatively mild (Figure 1A): while the serum insulin concentrations of ad libitum-fed 8-week-old male s/s and db/db mice were similarly 10- to 15-fold elevated compared to +/+ animals (p = NS s/s versus db/db) and the blood glucose concentrations for s/s animals were elevated to approximately 15 mM, the blood glucose of db/db animals was increased to an average of >30 mM (p < 0.001 versus s/s). Note also that 4/10 db/db animals had blood glucose concentrations that read “Hi” on the glucometer, denoting glucose concentrations >35 mM, although we recorded these as 35 mM when calculating average blood glucose concentrations. Interestingly, while not floridly hyperglycemic, db/+ animals displayed increased circulating insulin (p < 0.05) and tended to have higher glucose levels (trend, p = 0.1) compared to s/+ animals with similarly elevated feeding and adiposity. Furthermore, ob/ob and s/s littermates on the segregating C57Bl/6;129 background from compound heterozygote intercrosses yielded similar data as C57Bl/6 db/db and s/s animals: db/ob animals were dramatically more hyperglycemic than s/s mice, although db/ob and s/s animals were similarly hyperinsulinemic (Figure 1B). These data confirm that the differences observed between s/s mice and mice devoid of all leptin signals are independent of genetic background.

Similarly, while s/s animals display increased fasting blood glucose and a trend toward glucose intolerance during intraperitoneal glucose tolerance test (IPGTT) compared to +/+ animals (Figures 1C and 1D), the fasting glucose concentrations of db/db mice were further elevated, and the area under the IPGTT curve was increased by 40% for db/db compared to s/s (p = 0.05) as well as ++/+ (p < 0.01) animals. Furthermore, s/s mice were significantly more sensitive to insulin at short times in an intraperitoneal insulin tolerance test than were db/db animals (Figure 1E).

In aggregate, these data suggested that while the disruption of LRβ/STAT3 signaling in s/s animals results in increased adiposity and insulin resistance, LRβ/STAT3-independent signals that are intact in s/s but not in db/db animals also contribute to glycemic control. While it is formally possible that the modest but statistically significant difference in adiposity between s/s and db/db animals (Bates et al., 2003; and see Figure 3) could contribute to the observed differences in glycemic control between these two genotypes, the finding that s/s animals fail to progress to further elevated blood glucose concentrations when followed to 24 weeks of age (Figure 1F) while continuing to increase in weight (not shown) suggested to us that this effect is independent of adiposity.

Similar activation of the HPA axis in s/s and db/db mice

Since db/db (and ob/ob) animals are known to have increased activity of the hypothalamic-pituitary-adrenal (HPA) axis and this HPA axis hyperactivity contributes to their insulin resistance (Wittmers and Haller, 1983), we examined HPA axis activity in s/s and db/db animals to determine whether LRβ/STAT3-independent signals in s/s animals might suppress the HPA axis and thereby improve glycemic control in s/s compared to db/db mice. We thus measured serum corticosterone levels and plasma ACTH levels in s/s, and db/db mice that were accustomed to daily handling (Figure 2). Serum corticosterone levels were similarly elevated by 2.5-fold in s/s and db/db mice compared to +/+ mice (Figure 2A). In spite of this elevation of corticosterone levels, plasma ACTH levels were elevated by 10% in the s/s and db/db mice compared to +/+ animals (Figure 2B). This elevated ACTH in the face of hypercorticosternedia indicates inappropriate central activation of the HPA axis in s/s and db/db mice. Since both s/s and db/db mice possess similarly elevated HPA axis activity, these data demonstrate that LRβ/STAT3 signaling is crucial for the regulation of HPA activity by leptin and further suggest that differences in glucose homeostasis between s/s and db/db animals are not attributable to differences in glucocorticoid-induced insulin resistance.

Effects of pair feeding on adiposity and glycemic control in s/s and db/db mice

Since adiposity as well as central leptin action contributes to the regulation of glucose homeostasis and since s/s mice display slightly reduced adiposity compared to db/db animals (Bates et al., 2003), we undertook a pair-feeding regimen for s/s and db/db mice to explore the relative contributions of feeding/adiposity-dependent and -independent alterations to glucose homeostasis in these animals. We thus fed s/s and db/db mice the average amount of food consumed by age-matched wild-type mice from the time of weaning. While ad libitum-fed db/db mice displayed an almost 2-fold increase in food intake compared to +/+ mice (p < 0.001) and tended to eat slightly (5%) more than s/s mice (Figure 3A), there was no statistically significant difference in 24 hr food intake in s/s compared to db/db mice. Pair feeding of s/s and db/db animals resulted in a 25%–30% decrease in body weight compared to ad libitum-fed animals by six weeks of age (p < 0.01 versus ad libitum), although pair-fed (PF) s/s and db/db animals weighed substantially more than the wild-type animals to which they had been pair fed (p < 0.01), consistent with their increased metabolic efficiency (Figure 3B; Bates et al., 2004). While not different in body weight through six weeks of age, PF s/s animals weighed slightly more than PF db/db animals by 8 weeks of age (p < 0.05), consistent with the improved insulin-mediated energy storage in s/s animals (see discussion below).

While serum leptin levels for s/s and db/db mice trended down following pair feeding, the reduction in leptin levels was not statistically significant compared to ad libitum-fed animals, suggesting that the secretion of leptin by adipose tissue may be near maximal at the levels of adiposity achieved by these
Figure 1. Glucose homeostasis in mice with disrupted leptin receptor signaling

A) Blood glucose and serum insulin in ad libitum-fed 8-week-old male C57B/6 animals of the following genotypes +/+, s/s, db/+ and db/db mice. Mean blood glucose and serum insulin levels are plotted ±SEM; n = 8–11 per genotype.

B) Glucose and insulin in ad libitum-fed C57;129 animals. Blood glucose and serum insulin levels were measured in 8-week-old ad libitum-fed male control (?/+), s/s, and ob/ob mice on the same C57;129 mixed genetic background and mean values ±SEM are plotted; n = 8 per genotype.

C) Glucose tolerance. Male +/+, s/+, s/s, db/+ and db/db mice were fasted overnight and the GTT performed at 9:30 a.m. Following an initial blood glucose reading, a bolus of sterile glucose was administered at time zero and blood glucose measurements taken at timed intervals. Average glucose reading at each time is plotted for each genotype ±SEM; n = 8–20 per genotype.

D) Area under the GTT curve was calculated for each animal in (C) and averages for each genotype are plotted ±SEM.

E) Insulin tolerance test. Male s/s and db/db mice were fasted overnight and the ITT performed at 9:30 a.m. Following an initial blood glucose reading, a bolus of insulin was administered at time zero and blood glucose measurements taken at timed intervals. Average glucose reading at each time is plotted for each genotype ±SEM; n = 8 per genotype. F) Effect of age on glycemic control. Ad libitum-fed male +/+, s/+, s/s, db/+ and db/db mice were monitored longitudinally from 8–24 weeks of age for blood glucose and serum insulin. Average values for each genotype at each age are plotted ±SEM; n = 6–13 per genotype, except for db/db mice, which began to die at age 9 weeks; numbers of surviving db/db animals are indicated in parentheses above the error bars for these weeks. All panels: +/+(filled circles), s/s(filled squares), s/+(empty squares), db/db or ob/ob (filled triangles), and db/+ (empty triangles). Bars with different letters are statistically different (p < 0.01).

animals (Figure 3C). Analysis by DEXA of body composition of 8-week-old animals demonstrated that while pair feeding db/db animals to +/+ levels decreased percent body fat compared to ad libitum-fed db/db animals and equalized the percent adiposity of the PF s/s and db/db animals, the PF animals still displayed percent adiposity levels more than twice those of wild-type animals (Figure 3D; approximately 3-fold elevated total fat mass) and lean body mass that was similar or reduced.
compared to wild-type animals (data not shown). Thus, these data are consistent with the increased metabolic efficiency of s/s and db/db animals (Bates et al., 2004), resulting in increased fat storage compared to wild-type animals with equivalent caloric intake.

Caloric restriction delayed by 1–2 weeks but did not prevent the onset of hyperglycemia in db/db animals, while it succeeded in normalizing blood glucose levels in PF s/s animals with similar levels of body fat as the PF db/db mice (Figure 4A), suggesting that the differences in glycemic control observed between s/s and db/db animals were independent of differences in feeding and adiposity. Longitudinal analysis of serum insulin levels in these animals demonstrated the reduction of circulating insulin levels in PF s/s and PF db/db animals by caloric restriction (Figure 4B), consistent with a reduction of insulin resistance due to decreased adiposity. These data also demonstrated dramatically increased insulin resistance in PF db/db compared to PF s/s animals from the time of weaning, as gauged by the maintenance of glycemic control in the PF s/s animals at insulin levels lower than the levels that failed to maintain glycemic control in the db/db animals. Indeed, in the 8-week-old PF animals, lower levels of circulating insulin in PF s/s than PF db/db animals resulted in normoglycemia for PF s/s animals, while the higher levels of insulin in PF db/db mice failed to control blood glucose levels in the normal range. These data suggest increased insulin resistance in db/db animals compared to s/s animals independent of any differences in food intake or adiposity. While the preceding data clearly demonstrate increased insulin resistance in db/db than s/s animals, the reduction of serum insulin levels in PF db/db compared to ad libitum-fed db/db mice at 8 weeks of age in the face of the continued hyperglycemia of PF db/db animals could indicate an additional defect in insulin production in db/db animals.

Islet function in s/s and db/db mice

In order to formally address whether a decrease in insulin production contributes to the lower insulin levels and poor glycemic control in the PF db/db mice, we assessed circulating insulin C-peptide levels in these mice at 8 weeks of age (Figure 4C). Insulin C-peptide levels were increased and highly variable in ad libitum-fed s/s and db/db animals compared to controls, consistent with the high variable levels of insulin secretion that likely result from the increased amount and frequency of feeding in these animals. In contrast, the morning C-peptide levels in the PF s/s mice, while elevated approximately 2-fold compared to +/+ animals, were less variable, consistent with the consumption of most of their calories at the onset of the dark cycle and the subsequent absence of recent feeding. PF db/db mice displayed insulin C-peptide levels twice as high as those of s/s animals, suggesting that the decrease in circulating insulin in PF db/db mice compared to s/s mice does not reflect decreased insulin production in the db/db animals but increased clearance and that insulin production is actually 2-fold higher in PF db/db than in PF s/s mice.

In order to more carefully examine the insulin-producing islets in these mouse models, we examined their size and morphology in histologic sections of pancreata from 8-week-old animals (Figure 5). Assessment of islet area and morphology revealed a 50%–80% increase in islet area in both s/s and db/db mice compared to +/+ mice, which is consistent with the increased demands for insulin production resulting from the insulin resistance of these animals. While the differences in islet area between ad libitum-fed s/s and db/db mice were not significant (trend, p = 0.1), the islet area of PF s/s animals was significantly smaller than of db/db mice under all feeding conditions (p < 0.05), consistent with a greater demand placed...
Figure 4. Glycemic control in mice with leptin receptor mutations. Morning blood glucose (A) and serum insulin (B) concentrations were determined weekly for animals of all genotypes and feeding conditions. Serum insulin C-peptide concentrations were determined in 8-week-old animals from all genotypes and feeding conditions (C). All values represent mean ± SEM for n = 7–8 animals for each genotype and condition. Genotypes as indicated in (C); for all other panels: +/+ (filled circles), s/s (filled squares), PF s/s (empty squares), db/db (filled triangles), and PF db/db (empty triangles). Bars with different letters are statistically different (p < 0.01).

Hepatic steatosis and gene expression in s/s and db/db mice

In aggregate, these data suggest that there are significant differences in glucose homeostasis in s/s compared to db/db mice that cannot be attributed to differences in adiposity or insulin production between these two models. Others have suggested that central leptin action regulates hepatic function and insulin sensitivity (Liu et al., 1998; Kahn and Rossetti, 1998; Rossetti et al., 1997; Asilmaz et al., 2004), and we hypothesized that some of these actions of leptin might be mediated by LRB/STAT3-independent signals. We therefore examined hepatic steatosis and the hepatic mRNA expression of a number of genes that have been reported by others to be regulated by leptin treatment (Figure 6). As expected, total liver weight and triglyceride content was increased in ad libitum-fed s/s and db/db mice, in agreement with the known hepatic steatosis in db/db mice (Figures 6A and 6B). Pair feeding normalized the weight of s/s and db/db mice and reduced the liver triglyceride content to near normal, suggesting that a significant portion of the hepatic steatosis of these models could be accounted for by excessive caloric intake and that differences in liver triglyceride content could not account for differences in glycemic control between the PF s/s and PF db/db mice.

To further assess hepatic function, we measured mRNA expression for several key regulators of hepatic metabolic function using semiquantitative RT-PCR (Figures 6C–6F). We observed that IRS-2 mRNA was decreased to near or below the range of detection in both s/s and db/db mice compared to wild-type animals but that IRS-2 mRNA expression was normalized or enhanced by pair feeding in each of these models (Figure 6C), suggesting that recent feeding and/or insulin action regulates the expression of IRS-2 mRNA, as opposed to a primary effect of leptin (i.e., independent of feeding; Shimomura et al., 1999). Although liver PEPCK mRNA expression of an ad libitum-fed db/db mice trended slightly up, it was not significantly different from wild-type mice, nor was PEPCK mRNA expression altered in the liver of PF s/s animals (Figure 6D). The PEPCK mRNA expression in PF db/db animals was significantly elevated (p < 0.01) compared to wild-type animals, however. In contrast, the ad libitum-fed s/s animals displayed suppressed PEPCK mRNA levels compared to other groups. Notably, PEPCK mRNA expression was appropriately decreased in ad libitum-fed s/s animals that feed during the light as well as the dark cycle. This appropriate suppression of PEPCK mRNA in the ad libitum-fed s/s animals is consistent with increased hepatic insulin sensitivity in these animals compared to db/db animals as opposed to an effect of leptin, per se, since levels increased during the food restriction associated with pair feeding.

Steroyl CoA desaturase-1 (SCD-1) is a leptin-suppressed target gene in the liver that may contribute to overall energy homeostasis by regulating energy expenditure (Coen et al., 2002). SCD-1 mRNA expression was elevated compared to +/+ mice in db/db and s/s animals independently of feeding status, consistent with the regulation of this gene by leptin in a manner independent of nutritional status and independently of LRB/STAT3 signaling (Figure 6E). Thus, while this gene is regulated by leptin action independently of body adiposity, it is not responsible for the differences in insulin sensitivity between s/s and db/db mice, consistent with the recent findings of others (Asilmaz et al., 2004).
Pancreas morphology was assessed by evaluating at least three sections (7 microns thick) cut 150 microns apart from each pancreas from 8-week-old male mice. Sections were immunostained with a cocktail of antibodies against non-β cell hormones (glucagon, somatostatin, and pancreatic polypeptide) as described earlier (Kulkarni et al., 1999). β cell area was evaluated using Openlab image analysis software for calibrating the magnification for each micrograph (Image Pro Plus; Media Cybernetics, Silver Spring, Maryland). Images of sections were captured using a BX60 microscope (Olympus) equipped with a U-PMTVC video adaptor and an HV-C20 TV camera (Hitachi, Tokyo). The percentage of cells positive for β or non-β cells was calculated and corrected for pancreatic weight. Panel: All values represent mean ±SEM for n = 5 animals for each genotype and condition. Genotypes as indicated. Bars with different letters are statistically different (p < 0.01).

The sterol regulatory binding protein SREBP1c isoform is predominantly found in liver and adipose tissue and mediates several transcriptional actions of insulin (Brown and Goldstein, 1997; Yahagi et al., 2002). As in ob/ob mice, we found that hepatic SREBP1 mRNA expression was significantly increased in db/db and s/s mice compared to wild-type; however, s/s mice had lower expression levels compared to db/db mice (Figure 6F). Pair feeding did not alter expression levels of SREBP1 mRNA despite reducing serum insulin levels in these mice. These data suggest insulin-independent control of SREBP1 mRNA expression and are consistent with the improved insulin sensitivity of s/s mice compared to db/db mice.

LRβ/STAT3 signaling in the regulation of glucose homeostasis

Others have shown that leptin regulates insulin sensitivity by two methods: one dependent upon adiposity/feeding, and the other via a CNS-mediated pathway independent of adiposity and feeding (Petersen et al., 2002; Oral et al., 2002; Shimomura et al., 1999; Colombo et al., 2002; Ebihara et al., 2001; Liu et al., 1998; Burcelin et al., 1999; Kamohara et al., 1997; Barzilai et al., 1999). Our present results are consistent with this model and further suggest that LRβ/STAT3 signaling is critical for the feeding/adiposity-dependent regulation of glucose homeostasis, in line with the requirement for this signal in the regulation of feeding and energy expenditure (Bates et al., 2003, 2004): glucose homeostasis in freely feeding obese s/s mice, though improved compared to db/db mice, is impaired.

Although insulin resistance is not entirely ameliorated in the PF s/s animals (as evidenced by their increased circulating insulin and insulin C-peptide levels), this is consistent with the increase in circulating glucocorticoids in the s/s animals and with the increased adiposity of the PF s/s animals due to decreased energy expenditure of these animals compared to wild-type mice (Bates et al., 2004). PF s/s mice display similar percent adiposity as PF db/db animals; at 8 weeks of age the
Liver composition and gene expression

Livers from ad libitum and PF animals were removed following sacrifice of 8-week-old animals and weighed (A). Similar portions of each liver were then dissected and frozen for the determination of triglyceride content (B) or for the isolation of RNA for the examination of gene expression (C–F). Expression of mRNA for IRS-2 (C), PEPCK (D), SCD-1 (E), and SREBP1c (F) was determined by semiquantitative RT-PCR using the resulting liver RNA. All data are shown as mean ±SEM; n = 7–8 per genotype and condition. mRNA expression is reported in arbitrary units (A.U.) for comparison within each panel. Genotypes as indicated. Bars with different letters are statistically different (p < 0.01).

PF s/s body mass and thus total adipose tissue mass is also higher than for PF db/db mice. The divergence of weight between PF s/s and PF db/db mice after 6 weeks of age may reflect caloric loss by the PF db/db mice due to glycosuria. The normoglycemia of PF s/s but not PF db/db mice (with identical total and hepatic adiposity) thus suggests that LRb/STAT3-independent signals constitute the majority of the feeding/adiposity-independent regulation of glucose homeostasis by leptin. The appropriate suppression of hepatic PEPCK mRNA expression in s/s mice is similarly consistent with this conclusion. The differences in glycemic control between s/s and db/db animals cannot be attributed to decreased insulin production in db/db animals since islet area and insulin production (reflected in insulin C-peptide levels) are actually higher in the db/db cohort than in s/s animals. While it is theoretically possible that some of the observed differences between PF s/s and PF db/db animals could reflect secondary effects of hyperglycemia in the PF db/db mice, it is clear that important differences between these two lines must exist to explain the genesis of the hyperglycemia in the PF db/db mice; many or all of the differences we have observed could reasonably reflect these primary alterations in insulin action and glycemic control.

It is not clear whether the feeding/adiposity-independent regulation of glycemic control by leptin represents an increase in insulin sensitivity per se or whether leptin acts to regulate hepatic glucose output independently of any effect on insulin signaling. It is clear from the work of others that this leptin effect is mediated via the CNS, as central manipulation of LRb expression or administration of leptin mediates similar effects (Cohen et al., 2001; Liu et al., 1998; Kowalski et al., 2001; Chua et al., 2004). Furthermore, recent work has suggested that the central effects of leptin on hepatic glucose production are mediated independently of the hypothalamic melanocortin system (Gutierrez-Juarez et al., 2004). Consistently, we demonstrated impaired hypothalamic proopiomelanocortin expression with relatively preserved repression of hypothalamic NPY expression in s/s mice (Bates et al., 2003). Since leptin also regulated the development and plasticity of the metabolic circuitry of the brain (Pinto et al., 2004; Bouret et al., 2004), it is also possible that alterations in either of these parameters between s/s and db/db mice may contribute to differences in glycemic control.

While it is theoretically possible that Tyr1138 of LRb may mediate signaling by an unknown STAT3-independent LRb signal-
ing pathway that would also be disrupted in s/s animals, there are no data to suggest that this is the case. Thus, in aggregate, these results suggest that while the LRb/STAT3 signaling pathway is required for the action of the hypothalamic melanocortin system and thereby the regulation of feeding, energy expenditure, and adiposity, LRb/STAT3-independent leptin signals (perhaps via the hypothalamic NPY system) primarily mediate the central and adiposity-independent effects of leptin on glycemic control. Since other growth factors and cytokines activate STAT3 in the brain, presumably contributing to the more severe phenotype of neuronal STAT3 knockout mice than that of s/s mice (Gao et al., 2004), the untested possibility exists that these LRb-independent STAT3 signals may also contribute to glycemic control.

As we grapple with the increasing incidence of the metabolic syndrome and type 2 diabetes in developed nations, it is crucial to understand the mechanisms contributing to the development of obesity and diabetes and to define the similarities and differences in the processes controlling obesity and diabetes. While defects in the β cell are likely to contribute to the onset of diabetes, our data demonstrate no differences in islet function between animals devoid of LRb/STAT3 signaling and animals lacking LRb entirely. It is clear, however, that abrogation of the LRb/STAT3 signal contributes to the development of hyperglycemia secondary to hyperphagia and obesity but also that the LRb/STAT3 pathway does not contribute to the adiposity-independent control of glycemic homeostasis. Rather, LRb/STAT3-independent leptin pathways contribute to the direct, feeding/adiposity-independent regulation of glycemic control; understanding these pathways will likely be important for defining the mechanisms contribute to the development of type 2 diabetes.

Experimental procedures

All animal experimentation was conducted in accordance with mandated standards of humane care, approved by the Institutional Animal Care and Use Committee. Mice were housed in individually ventilated cages on a 12 hr light-dark cycle with ad libitum access to LabDiet autoclavable laboratory mouse chow 5021, a high-calorie breeder chow, and water, unless otherwise indicated. C57BL/6 (six generations backcrossed) mice heterozygous for the lepr<sup>+</sup> mutation (s/+)) were bred to generate s/s and wild-type littermates for study. C57BL/6 db/+ animals obtained from the Jackson Laboratory (Bar Harbor, Maine) were intercrossed to generate db/db and db/+ animals for study. C57Bl/6 ob/+ animals were obtained from Jackson laboratories and intercrossed with s/+ animals on the segregating C57Bl/6;129 genetic background to generate N1F1 s/s, ob/+ compound heterozygotes that were subsequently intercrossed to generate N1F2 s/s and ob/ob littermate animals for study.

Glucose tolerance test

Mice were fasted overnight for 16 hr. At 9:30 a.m. an initial blood glucose reading was taken (Glucometer Elite) and mice were administered an intraperitoneal bolus of glucose (2 g/kg body weight). Whole venous blood was obtained from the tail vein to measure blood glucose 15, 30, 60, and 120 min after glucose administration.

Insulin tolerance test

Mice were fasted overnight for 16 hr. At 9:30 a.m. an initial blood glucose reading was taken (Glucometer Elite) and mice were administered an intraperitoneal bolus of insulin (4 units/kg body weight). Whole venous blood was obtained from the tail vein to measure blood glucose 15, 30, and 60 min after insulin administration.

Pair-feeding studies

All mice were weaned at 21 days of age and housed individually. Twenty-four hour food intake was monitored by weighing chow daily for male wild-type mice for 5 weeks (until 8 weeks of age). Each day s/s and db/db mice were provided the average amount of food consumed by age-matched wild-type mice. Body weight was assessed at 3, 4, 6, and 8 weeks of age and blood samples were obtained by tail-vein nick for the measurement of blood glucose, serum leptin, and serum insulin.

Body composition

8-week-old male ad libitum-fed mice were briefly anaesthetized with Avertin (tribromoethanol:tert-amyl alcohol, 0.015 ml/g, i.p.) and subject to dual energy X-ray absorptiometry (DEXA; Lunar PIXimus2 densitometer, GE Medical Systems, Madison, Wisconsin) for the analysis of fat and lean body mass.

Hormone measurement

For the measurement of serum insulin and leptin, blood was obtained by tail vein nick and serum was collected and stored at −20°C. Serum insulin and leptin concentrations were measured by ELISA (Crystal Chem, Illinois). For the measurement of parameters of the H-P-A axis, all mice were handled daily for at least 10 days prior to the day of the experiment. Mice were sacrificed at 10 a.m. by cervical dislocation and decapitated for the collection of trunk blood. For the measurement of corticosterone, serum was collected, stored at −20°C, and serum concentrations were assessed by RIA (ICN). For the measurement of ACTH, trunk blood was collected in microtainer spin tubes containing EDTA (Becton Dickinson, Franklin Lakes, New Jersey) and centrifuged. Plasma was collected and stored at −80°C and ACTH assessed by RIA (ICN). Insulin C-peptide was measured by RIA (Linco, St. Louis, Missouri) of serum obtained from terminal bleeds of 8-week-old male mice.

Liver analysis

Wet liver weight was measured on freshly dissected livers of 8-week-old male mice. The left lobe of the liver was snap frozen for later determination of triglyceride content and for the purification of RNA for gene expression analysis. Triglyceride content was assessed using the L-Type TG H N-(2-hydroxy-3-sulfopropyl)-3,5-dimetoxyaniline (HDAOS) biochemical assay using 50 mg frozen tissue (Wako Diagnostics, Richmond, Virginia). Briefly, tissue was homogenized in 5 ml of 150 mM sodium chloride, 0.1% Triton X-100, and 10 mM Tris (pH 8.0) and incubated at 60°C for 1 hr. Triglycerides were measured in 30 µl homogenate at a wavelength of 600 nm. Lipid standards of known concentration were used to generate a standard curve.

Liver gene expression

Total RNA was extracted from 50–100 mg liver samples using the Ultraspec RNA Isolation System (Biotex Inc., Houston, Texas) as described previously and DNase-treated using RQ1 RNase-free DNase (Promega, Madison, Wisconsin). For determination of relative RNA concentration, total liver RNA was subjected to automated fluorescent RT-PCR on an ABI 7700 (Applied Biosystems, Foster City, California) or MJ research Opticon (Waltham, Massachusetts). Primers used were: cttgagtttatgtagcagaaagtg (forward IRS-2), ttagagggctgattctgtgt (reverse IRS-2), TET-tcaacaagctggcctga-1gctg-mTT101194314 (probe IRS-2); ccccaaggatgagggtaat (forward Adipo-nectin Receptor 2). PEPCK was determined using a predesigned taqman primer/probe mix (Assays-on-Demand mM00440636_m1 Applied Biosys-tems). Primer/probe mix for SCD-1 and SREBP1 were obtained by submitting 300 bp sequence to Applied Biosystems Assays-by-Design service. Ribosomal RNA control primers and probes were as supplied by Applied Biosystems. Each predicted RT-PCR product spanned an intron/exon junction except for IRS-2, which is essentially intronless; RT-free controls were run for determinations of all mRNA species studied. Each RT-PCR reaction was determined to be in the linear range for quantitation by comparison to serial dilutions of input liver RNA.

Islet morphology

The pancreas was dissected from 8-week-old male mice and immediately immersed in Bouin’s solution overnight. Rinsed tissue was then fixed in...
10% buffered formalin until sectioning (7 microns thick cut 150 microns apart). Sections were immersed in a cocktail of antibodies against non-β cell hormones (glucagon, somatostatin, and pancreatic polypeptide) as described earlier (Kulkarni et al., 1999). β cell area was evaluated for three sections from each pancreas using Openlab image analysis software for calibrating the magnification for each micrograph (Image Pro Plus; Media Cybernetics, Silver Spring, Maryland). Images of sections were captured using a BX60 microscope (Olympus) equipped with a U-PMTVC video adaptor and an HV-C20 TV camera (Hitachi, Tokyo). The percentage of cells positive for β or non-β cells was calculated and corrected for pancreatic weight.

Acknowledgments
This work was supported by NIH DK57768 and 56731 (to M.G.M.) and an American Diabetes Association/European Association for the Study of Diabetes Transatlantic Fellowship (to S.H.B.).

Received: January 3, 2005
Revised: February 11, 2005
Accepted: February 18, 2005
Published: March 15, 2005

References


Kloek, C., Haq, A.K., Dunn, S.L., Lavery, H.J., Banks, A.S., and Myers, M.G., Jr. (2002). Regulation of Jak kinases by intracellular leptin receptor adaptor and an HV-C20 TV camera (Hitachi, Tokyo). The percentage of cells positive for β or non-β cells was calculated and corrected for pancreatic weight.


Kulkarni, R.N., Bruning, J.C., Winnay, N.J., Postic, C., Magnuson, M.A., and
ARTICLE


